

RESEARCH PAPER

# Response of barley plants to Fe deficiency and Cd contamination as affected by S starvation

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## Abstract

Both Fe deficiency and Cd exposure induce rapid changes in the S nutritional requirement of plants. The aim of this work was to characterize the strategies adopted by plants to cope with both Fe deficiency (release of phytosiderophores) and Cd contamination [production of glutathione (GSH) and phytochelatins] when grown under conditions of limited S supply. Experiments were performed in hydroponics, using barley plants grown under S sufficiency (1.2 mM sulphate) and S deficiency (0 mM sulphate), with or without Fe<sup>III</sup>-EDTA at 0.08 mM for 11 d and subsequently exposed to 0.05 mM Cd for 24 h or 72 h. In S-sufficient plants, Fe deficiency enhanced both root and shoot Cd concentrations and increased GSH and phytochelatin levels. In S-deficient plants, Fe starvation caused a slight increase in Cd concentration, but this change was accompanied neither by an increase in GSH nor by an accumulation of phytochelatins. Release of phytosiderophores, only detectable in Fe-deficient plants, was strongly decreased by S deficiency and further reduced after Cd treatment. In roots Cd exposure increased the expression of the high affinity sulphate transporter gene (*HvST1*) regardless of the S supply, and the expression of the Fe deficiency-responsive genes, *HvYS1* and *HvIDS2*, irrespective of Fe supply. In conclusion, adequate S availability is necessary to cope with Fe deficiency and Cd toxicity in barley plants. Moreover, it appears that in Fe-deficient plants grown in the presence of Cd with limited S supply, sulphur may be preferentially employed in the pathway for biosynthesis of phytosiderophores, rather than for phytochelatin production.

**Key words:** Iron deficiency, iron uptake, phytochelatins, phytosiderophores, Strategy II, sulphur deficiency, thiols.

## Introduction

Iron (Fe) acquisition in graminaceous plants is characterized by secretion of chelating compounds, phytosiderophores (PSs), and by specific uptake of Fe(III)–PS complexes.

Several studies have indicated a fundamental role for S metabolism in homeostasis and detoxification of heavy metals in plants (Na and Salt, 2011). Similarly, significant interactions between plant Fe acquisition mechanisms and external sulphate (SO<sub>4</sub><sup>2-</sup>) supply have been reported. For example, lower leaf Fe concentrations have been observed

in S-deficient plants as compared with the S-sufficient control (Astolfi *et al.*, 2003b). In addition, it was demonstrated that a low availability of sulphate could affect accumulation (Kuwajima and Kawai, 1997) and release (Astolfi *et al.*, 2006a) of PSs in Fe-deficient barley roots, associated with a possible impairment of Fe acquisition in these plants.

These results suggest that the requirement for S may be higher when plants are under Fe deficiency and, on the

Abbreviations: DMA, deoxymugineic acid; DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid); epiHMA, epi-hydroxymugineic acid; GSH, glutathione; MA, mugineic acid; NS, nutrient solution; PC, phytochelatin; PS, phytosiderophore; SPAD, Soil Plant Analysis Development.

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other hand, that plant responses to Fe deficiency are modified by S supply.

The presence of cadmium (Cd) in the environment is another factor causing a higher plant requirement for reduced S, due to the increased production of sulphur-containing metabolites, such as glutathione (GSH) and its derivatives (phytochelatins, PCs), required for Cd detoxification. PCs are a family of peptides characterized by repeated units of  $\gamma$ -glutamylcysteine followed by a terminal glycine (Gly):  $(\gamma\text{-Glu-Cys})_n\text{Gly}$ , with  $n=2\text{--}11$  (Grill *et al.*, 1985; Cobbett and Goldsbrough, 2002). This implies the importance of an adequate supply of reduced S as an essential factor for biosynthesis of PCs.

Cd contamination of soils is a worldwide problem, and can cause losses in agricultural yield and pose a potential health risk for humans. Some reports indicate Cd as one of the most bioavailable heavy metal in soils (Lee *et al.*, 1998). Major sources of pollution in agricultural soils are fertilizer (mainly phosphate) impurities, the use of sewage sludge, and atmospheric fall-out from industrial and urban activities (Tamaddon and Hogland, 1993; Merrington and Alloway, 1994; McLaughlin and Singh, 1999). Despite the fact that Cd has no plant nutritional function, it is readily taken up by plants mainly due to limited specificity of  $\text{Fe}^{2+}$  and  $\text{Zn}^{2+}$  transporters, and thus it can be rapidly accumulated. Cd, even at low concentrations, inhibits plant growth and also disturbs photosynthesis, carbohydrate metabolism, sulphate assimilation, and several enzyme activities (Clijsters and Van Assche, 1985; Sanità di Toppi and Gabbrielli, 1999). The root is the first target of Cd phytotoxicity, and the interactions between Cd and root cells lead to physiological alterations such as membrane damage and changes in enzyme activities, involved in uptake and transport of mineral nutrients (Rubio *et al.*, 1994; Ouariti *et al.*, 1997; Astolfi *et al.*, 2003, 2005).

Cd interference with the Fe assimilation process (Wallace *et al.*, 1992; Siedlecka, 1995) often results in 'induced Fe deficiency' despite satisfactory Fe availability (Marschner, 1995). On the other hand, it has been demonstrated that Fe deficiency results in increased uptake and accumulation of Cd (Siedlecka and Krupa, 1999).

These findings suggest that plant responses to Fe deficiency and Cd exposure seem to be related to S homeostasis, and are in both cases associated with a higher plant requirement for reduced S.

The focus of this study is the strategies adopted by barley plants to cope with both Fe deficiency and Cd contamination, when grown under conditions of limited S supply.

The production of compounds (GSH and PCs) involved in Cd detoxification and Fe acquisition (PS release) was determined, in addition to the evaluation of common growth parameters (biomass, leaf green values, and mineral concentrations) and an expression analysis of *HvST1*, *HvYS1*, and *HvIDS2* genes.

Understanding interactions of S homeostasis with Fe deficiency and Cd detoxification pathways may contribute to the development of biotechnological applications for alleviation of Fe deficiency and Cd contamination, with valuable effects on agricultural and human health problems.

## Materials and methods

### Growing conditions

Because of the higher release rate of PSs by barley compared with other grasses, and thus a much easier detection, barley was preferred as the model plant.

Barley (*Hordeum vulgare* L. cv. Europe) seeds were germinated in moistened paper in the dark at 26 °C for 3 d. Seedlings were then transferred into plastic pots containing 2.2 l of nutrient solution (NS) (18 seedlings in each pot) and were cultured for 11 d, being exposed to S sufficiency (1.2 mM sulphate) and S deficiency (0 mM sulphate), with or without 0.08 mM  $\text{Fe}^{\text{III}}$ -EDTA. The composition of the NS (in mM) was:  $\text{K}_2\text{SO}_4$  0.7,  $\text{MgSO}_4$  0.5,  $\text{Ca}(\text{NO}_3)_2$  2.0,  $\text{KCl}$  0.1,  $\text{KH}_2\text{PO}_4$  0.1,  $\text{H}_3\text{BO}_3$   $1 \times 10^{-3}$ ,  $\text{MnSO}_4$   $1 \times 10^{-3}$ ,  $\text{CuSO}_4$   $2.5 \times 10^{-4}$ ,  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$   $1 \times 10^{-5}$ , and  $\text{ZnSO}_4$   $1 \times 10^{-3}$  (Zhang *et al.* 1991). In S-deficient NS, sulphate salts ( $\text{K}^+$ ,  $\text{Mn}^{2+}$ ,  $\text{Zn}^{2+}$ , and  $\text{Cu}^{2+}$ ) were replaced by appropriate amounts of the corresponding chloride salts ( $\text{K}^+$ ,  $\text{Mn}^{2+}$ ,  $\text{Zn}^{2+}$ , and  $\text{Cu}^{2+}$ ). The NS was continuously aerated and renewed every 2 d. Plants were grown in a growth chamber under controlled climatic conditions: light  $200 \mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetic photon flux (PPF) with a 14 h/10 h day/night regime (27/20 °C day/night temperature cycling; 80% relative humidity). After 11 d,  $\text{CdSO}_4$  or  $\text{CdCl}_2$  (final concentration 0.05 mM) was added to the NS and the plants were analysed 24 h or 72 h after Cd application.

### GSH and PC quantification

Root and shoot samples (300 mg fresh weight each) were homogenized in a mortar in the presence of ice-cold 5% (w/v) 5-sulphosalicylic acid containing 6.3 mM diethylenetriaminepentaacetic acid (DTPA), following the method of De Knecht *et al.* (1994) with minor modifications. Briefly, after centrifugation at 10 000 g for 10 min at 4 °C, the supernatants were filtered through Minisart RC4 0.45  $\mu\text{m}$  filters (Sartorius, Goettingen, Germany) and immediately assayed by HPLC (model 200, Perkin-Elmer, Norwalk, CT, USA). Thiol peptide compounds (GSH and PCs) were separated through a reverse-phase Purosphere  $\text{C}_{18}$  column (Merck GmbH, Darmstadt, Germany), by injecting 200  $\mu\text{l}$  of each extract. Separation was achieved by means of an HPLC integrated system (Series 200 pumps, PerkinElmer, MA, USA) in a 0–26% acetonitrile gradient (Merck, Germany), containing 0.05% trifluoroacetic acid at a flow rate 0.7 ml  $\text{min}^{-1}$ . Thiol peptide compounds were detected by post-column derivatization with 300  $\mu\text{M}$  Ellman's reagent [5,5'-dithio(2-nitrobenzoic acid)] at 412 nm (Series 200 detector, PerkinElmer). Identification of GSH and individual PCs was based on the comparison of their retention times with standard GSH (Merck) and PC samples from *Silene vulgaris*. A calibration curve for standard -SH groups was used for quantification of thiol peptides in extracts.

### Collection of root exudates and determination of PS release

Release of PSs from S-sufficient and S-deficient barley plants was analysed by determining the PS concentration in root washings. Barley plants were removed from the NS at 2 h after the onset of the light period and the roots were washed twice for 1 min in deionized water. Root systems were submerged in 500 ml of deionized water for 3 h with continuous aeration. Thereafter, Micropur (10 mg  $\text{l}^{-1}$ ) (Roth, Karlsruhe, Germany) was added to prevent microbial degradation of PSs. The PS concentration in root washings was determined by direct injection (20  $\mu\text{l}$ ) using the anion exchange HPLC system with post-column orthophthaldialdehyde (OPA) derivatization described by Neumann *et al.* (1999).

### Total RNA extraction and RT-PCR analysis

Isolation of total RNA from the roots of barley plants was performed using the Trizol<sup>®</sup> reagent system according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

A 1 µg aliquot of DNase-treated RNA was reverse transcribed by M-MLV (H-) Reverse Transcriptase (Invitrogen) to synthesize the first strand of cDNA. A 1 µl aliquot of the oligo(dT)12-primed first-strand cDNA samples was subjected to PCR using reverse and forward primers designed on the basis of the published *HvST1* gene sequence (accession no. Q43482) and that amplify a fragment of 699 bp. Primers were as follows: *HvST1*-1 primer, 5'-CGGATTCTTCAGGCTAGGGT-3'; and *HvST1*-2 primer, 5'-GCCACCATTCTTTGTTCC-3'. In addition, 1 µl of the oligo(dT)12-primed first-strand cDNA samples was subjected to PCR using reverse and forward primers designed by Hodoshima *et al.* (2007) to amplify specifically *HvYSL* and *HvIDS2* cDNA. Primers were as follows: *HvYSL*-1 primer, 5'-CGCCTCCAGTGTAGAAC-CAT-3'; and *HvYSL*-2 primer, 5'-TACCGATCAAGGGGACAA GA-3'; *HvIDS2*-1 primer, 5'-CTGATTGATTGAGGAATCCAG A-3'; and *HvIDS2*-2 5'-GAGTTCGTCAGCGAGGACA-3'. The level of 18S gene expression was used as a control for quantification. The PCR program for *HvST1* and 18S (control) was: one initial cycle of 94 °C for 5 min, followed by cycles of 93 °C and 55 °C for 1 min each and 72 °C for 2 min for each cycle, with 40 and 30 cycles for *HvST1* and 18S (control), respectively, all followed by a final extension at 72 °C for 7 min. PCR conditions for *HvYSL* and *HvIDS2* were: 94 °C and 60 °C for 1 min each and 72 °C for 45 s for each cycle, plus a final extension at 72 °C for 7 min. The number of PCR cycles was adjusted to obtain detectable amounts of amplicons without reaching signal saturation, which was accomplished with 32 cycles. All semi-quantitative RT-PCRs were performed in duplicate. RT-PCR amplification products were separated electrophoretically on 1% (w/v) agarose gels and stained with ethidium bromide.

#### Other measurements and statistics

Chlorosis scoring in attached leaves was conducted by a chlorophyll meter reading (SPAD) portable apparatus (Minolta Co., Osaka, Japan) using the first fully expanded leaf from the top of the plant.

To determine total S concentration, 1 g of each leaf or root sample was dried at 105 °C and then ashed in a muffle furnace at 600 °C. The ashes were dissolved in 10 ml of 3 M HCl and filtered through Whatman No. 42 paper. In contact with BaCl<sub>2</sub>, a BaSO<sub>4</sub> precipitate is formed which is determined turbidimetrically (Bardsley and Lancaster, 1962).

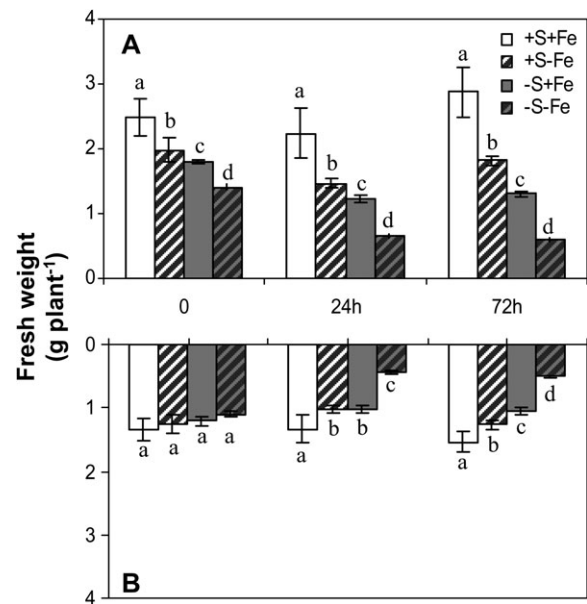
To determine the Fe and Cd concentration, leaf or root tissues were oven-dried at 80 °C to constant weight and thereafter ashed at 550 °C for 6 h in a muffle furnace. The ashes were dissolved in 1 M HCl and analysed by inductively coupled plasma atomic emission spectrometry in an ICP-AES instrument (VISTA MPX, Varian, Torino, Italy). Before Fe determination, the root extraplasmatic Fe pool was removed by 1.2 g l<sup>-1</sup> sodium dithionite and 1.5 mM 2,2'-bipyridyl in 1 mM Ca(NO<sub>3</sub>)<sub>2</sub> under N<sub>2</sub> bubbling according to the method described by Bienfait *et al.* (1985); the treatment was repeated three times.

Each reported value represents the mean ±SD of measurements carried out in triplicate and obtained from four independent experiments. Statistical analyses of data were carried out by analysis of variance (ANOVA) tests with the GraphPad InStat Program (version 3.06). Significant differences were established by post-hoc comparisons (HSD test of Tukey) at *P* < 0.01 or *P* < 0.05.

## Results

### Plant growth and concentrations of S, Fe, and Cd

After 14 d of culture, barley plants showed changes in some macro-indices (growth and SPAD readings), depending on the nutritional regime. Figure 1 illustrates that plants grown



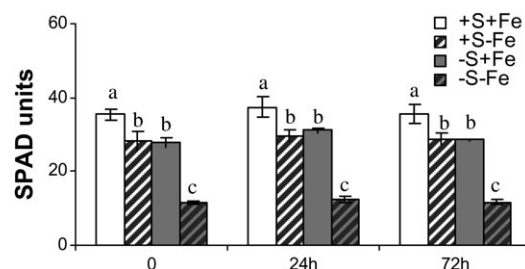
**Fig. 1.** Shoot (A) and root (B) fresh weight of S-sufficient and S-deficient barley plants grown for 11 d with (+Fe) or without (–Fe) 0.08 mM FeIII-EDTA exposed to 0.05 mM Cd for 24 h or 72 h (compared with 0 mM Cd). Data are means ±SD of four independent replications. Significant differences between samples are indicated by different letters (*P* < 0.01) (*n*=4).

under S starvation (0 mM sulphate) showed a significant decrease in fresh weight of both leaves and roots. The reduction in fresh weight was ~30% in leaves and only 10% in roots. Particularly in shoots, S starvation decreased the fresh weight more than Fe starvation, even if the shoot fresh weight of Fe-deficient plants was generally significantly lower compared with the respective Fe-sufficient control. Moreover, the addition of Cd to the nutrient solution at a concentration of 0.05 mM did not significantly affect the growth rate of S-sufficient plants, whereas it significantly reduced shoot and root biomass under S limitation.

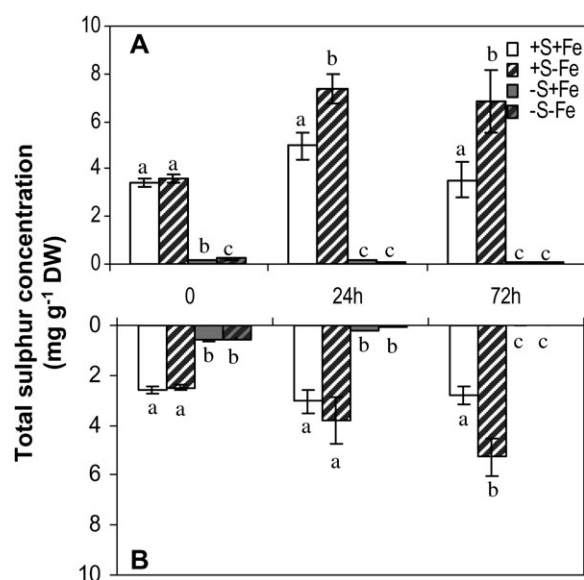
As regards SPAD readings (Fig. 2), young developing leaves from 14-day-old barley plants, grown without sulphate in NS, exhibited visible chlorosis (20% lower SPAD units). Fe deficiency led to severe leaf chlorosis at harvest, with a 20–70% decrease in SPAD readings, depending on S availability. Interestingly, the decrease in chlorophyll concentration was almost the same between +S–Fe and –S+Fe leaves. The results also showed that treatments with Cd did not significantly affect leaf chlorophyll concentration.

Analysis of the leaf and root mineral composition showed that the S concentration in S-starved plants was very low compared with S-sufficient plants (–96% and –75% in leaves and roots, respectively) (Fig. 3). Imposition of Fe deficiency on S-deficient plants (time 0) resulted in an increase in the leaf S concentration which was significantly higher than that of the Fe-sufficient plants, showing an ~45% increase with respect to the control. Also in S-sufficient plants Fe deficiency resulted in an increase in leaf S concentration, although to a lesser extent than in plants grown without





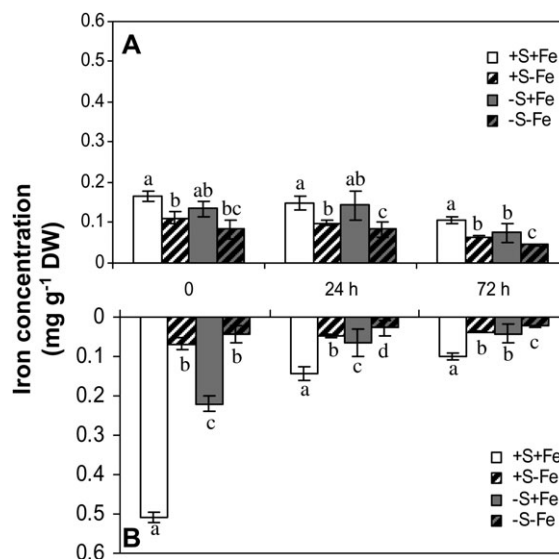
**Fig. 2.** Values of the SPAD index of S-sufficient and S-deficient barley plants grown for 11 d with (+Fe) or without (-Fe) 0.08 mM FeIII-EDTA exposed to 0.05 mM Cd for 24 h or 72 h (compared with 0 mM Cd). Data are means  $\pm$ SD of four independent replications. Significant differences between samples are indicated by different letters ( $P < 0.001$ ) ( $n=4$ ).



**Fig. 3.** Total S concentration in shoots (A) and roots (B) of S-sufficient and S-deficient barley plants grown for 11 d with (+Fe) or without (-Fe) 0.08 mM FeIII-EDTA exposed to 0.05 mM Cd for 24 h or 72 h (compared with 0 mM Cd). Data are means  $\pm$ SD of four independent replications. Significant differences between samples are indicated by different letters ( $P < 0.001$ ) ( $n=4$ ).

sulphate. The total S concentration of roots was unaffected by Fe deprivation. Additionally, the results showed that the exposure of S-sufficient plants to Cd treatment greatly increased the root and leaf total sulphur concentration of Fe-deficient plants. In particular, an increase was observed in leaf S concentration by  $\sim 105\%$  and  $90\%$ , after 24 h and 72 h, respectively (Fig. 3A), and also of root S concentration by  $\sim 54\%$  and  $110\%$ , after 24 h and 72 h, respectively (Fig. 3B). Also in Fe-sufficient plants, Cd treatment resulted in an increase in leaf and root S concentration, although to a lesser extent than in Fe-deficient plants. On the other hand, in S-starved plants no increase in S accumulation was observed after Cd treatment.

Figure 4 shows that Fe deprivation obviously decreased leaf (Fig. 4A) and root (Fig. 4B) Fe concentration. Interestingly, S supply increased leaf tissue concentrations of

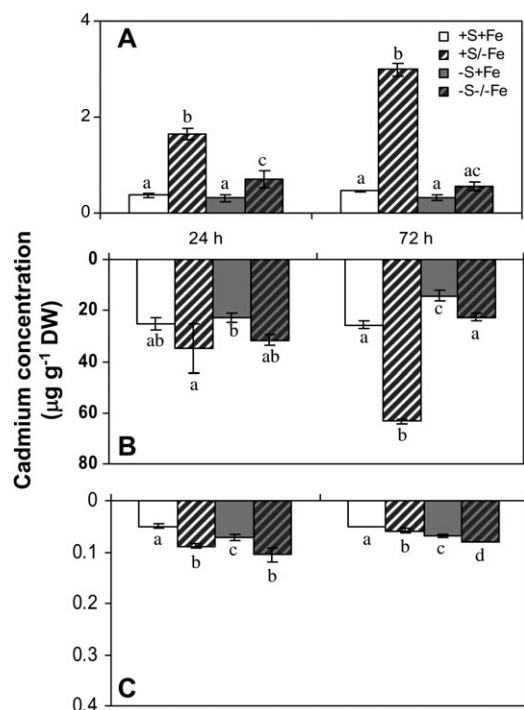


**Fig. 4.** Iron concentration in shoots (A) and roots (B) of S-sufficient and S-deficient barley plants grown for 11 d with (+Fe) or without (-Fe) 0.08 mM FeIII-EDTA exposed to 0.05 mM Cd for 24 h or 72 h (compared with 0 mM Cd). Data are means  $\pm$ SD of four independent replications. Significant differences between samples are indicated by different letters ( $P < 0.01$ ) ( $n=4$ ).

Fe by 23% and 40% as compared with S-deficient plants, at 0 h and 72 h, respectively (Fig. 4A). The relationship between S supply and Fe accumulation in roots showed a similar trend to that observed at leaf level, but the magnitude of the increase was more pronounced in roots than in shoots. Complete S starvation caused up to a 60% decrease in Fe accumulation in root tissues (Fig. 4B).

In each culture condition, Fe accumulation in both roots and shoots showed a trend towards a decrease with prolonged exposure of plants to Cd (Fig. 4A, B). At the leaf level, this reduction was  $\sim 40\%$  as compared with controls (point 0) 72 h after treatment onset (Fig. 4A). In roots, Fe accumulation was reduced by 80% and 50% after exposure of barley seedlings to Cd for 72 h, in -Fe and +Fe plants, respectively (Fig. 4B).

Cd addition to NS increased shoot and root Cd concentration, but there was significantly more Cd in the roots than in the shoots (Fig. 5A, B). In S-sufficient plants, Cd accumulation was higher in Fe-deficient plants, as compared with +Fe controls, the increase being greater in shoot than in roots. In particular, it was observed that addition of 0.05 mM Cd to Fe-deficient plants increased leaf Cd concentration by  $\sim 4$ - or 6-fold after 24 h and 72 h, respectively (Fig. 5A), and also increased root Cd concentration by  $\sim 40\%$  or 2-fold after 24 h and 72 h, respectively (Fig. 5A). Also in S-deficient plants, Fe starvation resulted in an increase in Cd concentration, although to a lesser extent than in plants grown at 1.2 mM sulphate. Figure 5B shows the total root Cd concentration, including the apoplastic as well as the symplastic concentrations. By remobilizing the apoplastic Cd concentration prior to analysis (Fig. 5C), the result was that root Cd concentrations were lower but, likewise, they increased equivalently in

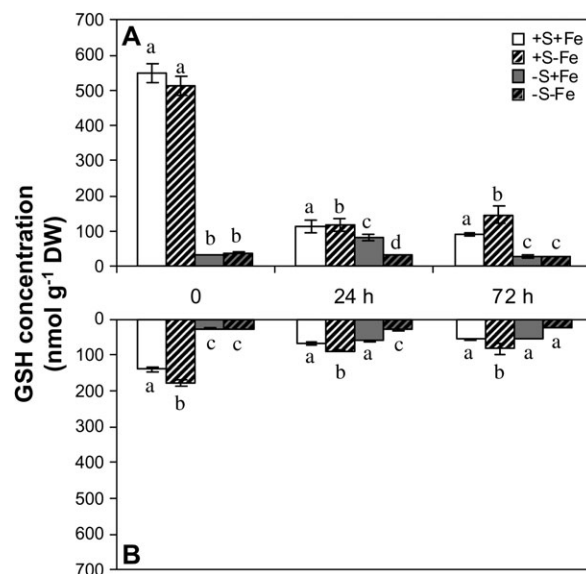


**Fig. 5.** Cadmium concentration in shoots (A) and in the root apoplast (B) and symplast (C) of S-sufficient and S-deficient barley plants grown for 11 d with (+Fe) or without (-Fe) 0.08 mM FeIII-EDTA exposed to 0.05 mM Cd for 24 h or 72 h (compared with 0 mM Cd). Data are means  $\pm$  SD of four independent replications. Significant differences between samples are indicated by different letters ( $P < 0.05$ ) ( $n=4$ ).

-Fe plants. In particular, this phenomenon occurred in a progressive manner on a time scale of few hours (from 24 h to 72 h).

#### Thiol peptide compounds

Although the GSH concentration was clearly related to S supply in both shoots and roots, it was unaffected by Fe deprivation in plants unexposed to Cd (point 0) (Fig. 6A, B). After the addition of Cd to S-sufficient plants, there was a sudden and sharp decrease in GSH levels (at least 50% in roots and up to 80% in shoots) in both Fe nutritional conditions. However, +S-Fe plants showed gradual increases in GSH levels compared with +S+Fe plants between 24 h and 72 h of treatment (Fig. 6A, B). On the other hand, Cd addition to -S plants led to a more than 2-fold higher GSH concentration than that found in -Cd plants (point 0) particularly within 24 h from treatment, but only in the +Fe condition. When Cd was added to -S-Fe plants, the amount of GSH gradually decreased by  $\sim 30\%$  in shoots and 20% in roots at 72 h of treatment. Consequently, it was observed that in S-deficient plants, incubation with Cd in combination with Fe deficiency (-S-Fe condition) caused depletion of the GSH concentration relative to the Cd-untreated control (-S+Fe condition). Summarizing these results, Cd exposure to both shoot and roots of -Fe plants (Fig. 5A, B) led to



**Fig. 6.** GSH production in shoots (A) and roots (B) of S-sufficient and S-deficient barley plants grown for 11 d with (+Fe) or without (-Fe) 0.08 mM FeIII-EDTA exposed to 0.05 mM Cd for 24 h or 72 h (compared with 0 mM Cd). Data are means  $\pm$  SD of four independent replications. Significant differences between samples are indicated by different letters ( $P < 0.05$ ) ( $n=4$ ).

higher GSH accumulation in S-sufficient plants, but not in S-deficient plants (Fig. 6A, B).

Cd treatment induced biosynthesis of PCs (PC<sub>2</sub> and PC<sub>3</sub> oligomers) in both shoots and roots of +S+Fe plants, and, at even higher levels, in +S-Fe plants as well (Fig. 7).

The highest total PC levels were found in roots, with concentration  $\sim 1.5$ - and 2-fold higher in +Fe condition and in -Fe condition, respectively, than those found in shoots after 72 h of Cd treatment (Fig. 7A, B). The shoot concentration of PCs decreased from 24 h to 72 h of Cd exposure ( $\sim 20\%$  in both +Fe and -Fe plants), whereas at root level it increased by  $\sim 40\%$  and 30%, respectively, in +Fe and -Fe plants. Exposure to Cd did not induce PC production in S-deficient plants, independently of Fe supply (Fig. 7).

#### PS release

Release of PSs was only detectable in Fe-deficient plants. In Cd-untreated plants, the rate of PS release was markedly decreased by S deficiency as already reported (Astolfi *et al.*, 2006a), reaching values up to 3-fold lower than control (+S) plants (Fig. 8). The rate of PS release in root exudates was further reduced following Cd treatment (Fig. 8). In particular, the effect of the treatment followed a similar pattern in all -Fe plants irrespective of their S supply and, as a consequence, the PS release rate maintained the same difference between +S and -S plants. Interestingly, the level of PSs released from roots of Fe-deficient plants was also qualitatively modified by both S starvation and Cd presence in the NS. In particular, before Cd treatment (time 0) only epi-hydroxymugineic acid (epiHMA) was detected in root

exudates, while deoxymugineic acid (DMA), mugineic acid (MA), and epiHMA were present in root exudates from plants treated with Cd for 24 h; DMA was only found in root exudates from these latter plants.

### Expression analysis

Expression of the high affinity sulphate transporter gene (*HvST1*) was up-regulated in roots of S-deficient plants, irrespective of the Fe nutritional status (Fig. 9, time 0). Some *HvST1* expression was also detectable in S-sufficient plants after Cd exposure (Fig. 9). In particular, transcript abundance became more pronounced with prolongation of the Cd treatment, especially in –Fe plants. Expression of both *HvYS1* and *HvIDS2*, two Fe deficiency-responsive genes, was obviously induced by Fe starvation (Fig. 9, time 0), but surprisingly also by Cd exposure in Fe-sufficient roots, although the expression level differed from that induced by Fe deficiency (Fig. 9).

## Discussion

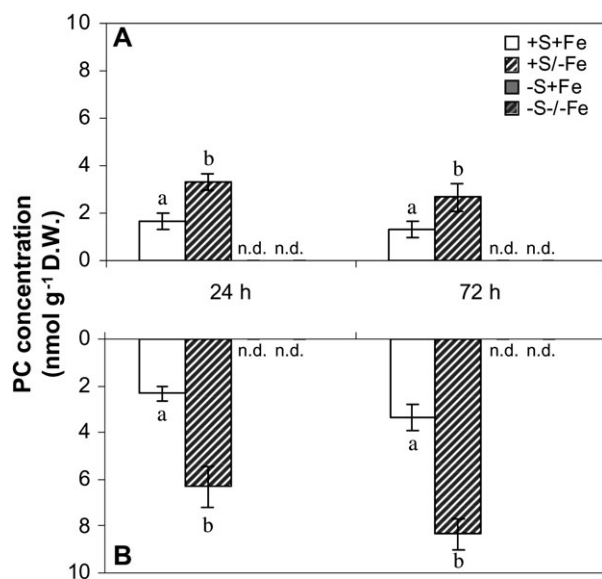
Leaf chlorosis is one of the earliest and distinct symptoms of S or Fe deficiency in plants (Marschner, 1995); it is also observed under Cd toxicity (Krupa and Baszynski, 1995). In the present study, it was found that leaf chlorophyll levels were markedly decreased after exposure of barley seedlings to single S or Fe deficiency or to the combined deficiency of the two nutrients, but they were hardly affected by 0.05 mM Cd treatment. On the other hand, a cumulative negative effect of overall treatments was observed when fresh weight accumulation was considered. These results are in line with the evidence that the interactions between Cd

and root cells lead to physiological alterations of plasma membrane properties (Astolfi *et al.*, 2003a, 2005), that in turn could affect water balance (Sanità di Toppi and Gabrielli, 1999). A failure in the water-absorbing capability of roots under Cd stress manifests its toxic effect, causing decreased water content in both root and shoot tissues (Fig. 1). Indeed, Cd treatment did not result in significant decreases in dry matter production of all barley plant parts (data not shown). At the same time, it must be emphasized that the Cd-induced inhibition of growth, resulting from the effect on fresh weight, was highest in Fe-deficient condition, confirming the results obtained by previous investigations in Cd-treated barley plants (Sharma *et al.*, 2004).

A higher accumulation of S in shoots was observed after exposure of barley seedlings to Fe deficiency in S-deficient conditions. This finding is consistent with previous results (Astolfi *et al.*, 2003b, 2006b). Higher S accumulation in both roots and shoots of barley seedlings was also found when they were exposed to Cd, as previously reported by Sharma *et al.* (2004). Furthermore, Cd-induced S accumulation was particularly evident in Fe-deficient plants. An enhanced accumulation of S in Fe-deficient barley plants under limited S supply was reported and related to the needs of sustaining the PS biosynthetic pathway (Astolfi *et al.*, 2006a, b). This observation is consistent with changes in the GSH level, stimulated by Fe depletion both in shoots and in roots, compared with +Fe plants. As a matter of fact, Cd treatment generates an additional S requirement, in order to support PC biosynthesis (Sanità di Toppi and Gabrielli, 1999). As a consequence of the simultaneous increases in S, Cd, and GSH contents in +S–Fe plants (Figs. 3, 5, 6), PC synthesis was higher in this treatment than in +S+Fe plants.

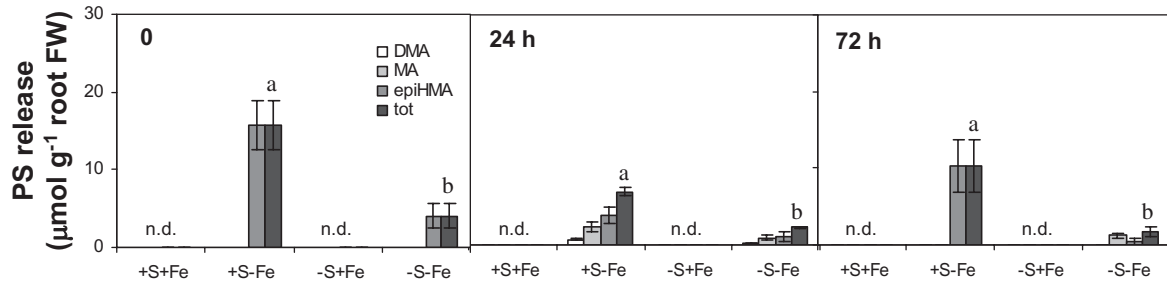
The nutrient uptake rate and transcription of transporter genes generally respond to the nutritional status of plants (Marschner, 1995). It is well known that the *HvST1* gene encoding a high affinity sulphate transporter is up-regulated in S-starved plants (Smith *et al.*, 1997; Hawkesford and Wray, 2000); it was also demonstrated that GSH acts as a repressor of the sulphate transporter (Lappartient and Touraine, 1996; Lappartient *et al.*, 1999). Here it is shown that the *HvST1* gene was up-regulated even in S-sufficient plants after Cd exposure (Fig. 9). This effect is probably due to a decreased GSH content (Fig. 5), as a consequence of synthesis of PCs (Fig. 6). This result supports the idea that barley plants try to compensate for GSH incorporation into PCs by means of increased S uptake (Nocito *et al.*, 2006). No PC accumulation could be observed in S-deprived plants upon Cd exposure, independently of the Fe nutritional status. Upon exposure to Cd, +S–Fe plants showed a higher Cd accumulation than +S+Fe plants (Fig. 3), thus explaining a higher GSH and PC content (Figs. 5, 6).

It was also found that plants exposed to Cd accumulated significantly more Cd in their roots than in their shoots. This finding is consistent with that reported in the literature and it is typical for most higher plants (Sandalio *et al.*, 2001; Kirkham, 2006; Ammar *et al.*, 2008). Interestingly, Cd accumulation in both shoots and roots was higher in Fe-deficient plants. Most studies concerning Cd–Fe interaction

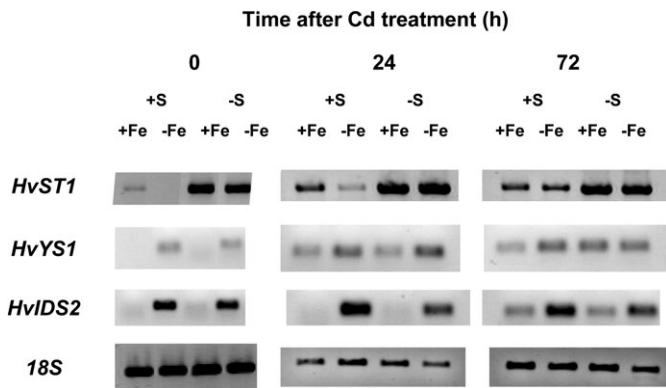


**Fig. 7.** Phytochelatin production in shoots (A) and roots (B) of S-sufficient and S-deficient barley plants grown for 11 d with (+Fe) or without (–Fe) 0.08 mM FeIII-EDTA exposed to 0.05 mM Cd for 24 h or 72 h (compared with 0 mM Cd). Data are means  $\pm$ SD of four independent replications. Significant differences between samples are indicated by different letters ( $P < 0.001$ ) ( $n=4$ ).





**Fig. 8.** Phytosiderophore release from roots of S-sufficient and S-deficient barley plants grown for 11 d with (+Fe) or without (-Fe) 0.08 mM FeIII-EDTA exposed to 0.05 mM Cd for 24 h or 72 h (compared with 0 mM Cd). Data are means  $\pm$  SD of four independent replications. Significant differences between samples are indicated by different letters ( $P < 0.001$ ) ( $n=4$ ).



**Fig. 9.** RT-PCR expression analysis of *HvST1*, a high affinity sulphate transporter gene, and *HvYS1* and *HvIDS2*, two Fe deficiency-responsive genes, in roots of S-sufficient and S-deficient barley plants grown for 11 d with (+Fe) or without (-Fe) 0.08 mM FeIII-EDTA exposed to 0.05 mM Cd for 24 h or 72 h (compared with 0 mM Cd). A 18S PCR product was used as a cDNA calibration control.

have been performed using plants characterized by a Strategy I-based response to Fe shortage (all plants except graminaceous monocots) and data are consistent with the involvement of the  $\text{Fe}^{2+}$  transporter (*IRT1*) in Cd uptake (Eide *et al.*, 1996; Cohen *et al.*, 1998; Korshunova *et al.*, 1999; Lombi *et al.*, 2002; Vert *et al.*, 2002). Furthermore, Yoshihara *et al.* (2006) demonstrated that not only Fe deficiency but also Cd exposure co-ordinately increased the expression of native genes such as *FRO1* and *IRT1* and the *HvIDS2pro::GUS* transgene in tobacco roots.

On the other hand, little is known about the response to Cd toxicity in Fe-deficient Strategy II plants (graminaceous monocots). Some authors reported that Fe deficiency did not increase shoot Cd concentration in wheat (Römheld and Awad, 2000) and maize (Hill and Lion, 2002), and discussed a potential role for PSs in Cd exclusion by Cd complexation (Hill and Lion, 2002). Other authors suggested the possibility of Cd uptake via the PS-dependent pathway (Sharma *et al.*, 2004). In contrast, Meda *et al.* (2007) recently demonstrated that uptake and translocation of Fe are decreased in maize plants exposed to Cd and that uptake of Cd-PS complexes is unlikely to be due to limited complex stability (Meda *et al.*, 2007). Furthermore, in the case of barley, the existence of

a transport system highly specific for the Fe(III)-PS complex was proven (Murata *et al.*, 2006). Thus, other mechanisms must be responsible for the observed response. The transcriptional up-regulation of the  $\text{Fe}^{2+}$  transporters (*OsIRT1* and *OsIRT2*) in rice plants under Fe limitation has been reported (Ishimaru *et al.*, 2006). Furthermore, more recently, the *IRT1* gene (*HvIRT1*) in barley roots was isolated and characterized (Pedas *et al.*, 2008). The predicted HvIRT1 protein exhibits 69% and 55% amino acid sequence identity to *OsIRT1* and *AtIRT1*, respectively, has the ability to transport  $\text{Fe}^{2+}/\text{Fe}^{3+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Zn}^{2+}$ , and  $\text{Cd}^{2+}$ , and, not least, the root expression of *HvIRT1* was induced by Fe deficiency (Pedas *et al.*, 2008). These studies seem to be in agreement with the present data, particularly as far as higher Cd accumulation in Fe-deficient plants is concerned. Thus, the increase in Cd concentration observed in Fe-deficient barley plants (Fig. 5) might conceivably be related to an up-regulation of the *IRT1* gene.

In leaves of Cd-exposed plants, the Fe concentration remained relatively stable up to 24 h, while showing a clear decrease at the end of the experiment. Several reports demonstrate that the Fe concentration is decreased under metal stress in plants, leading to the appearance of typical Fe deficiency symptoms (Wallace *et al.*, 1992; Siedlecka, 1995; Yoshihara *et al.*, 2006; Mendoza-Cozatl *et al.*, 2008). By contrast, other authors demonstrated that the Fe concentration in shoots of Cd-exposed barley plants (Hodoshima *et al.*, 2007) was unaffected after 2 d of treatment. Differences in growth conditions, Cd concentration, and treatment length might be the cause of the discrepancy among experimental data. Furthermore, it was found here that Fe accumulation was also reduced in roots exposure of barley seedlings to Cd and, more notably, this effect was also observed after desorption of apoplastic Cd (Bienfait *et al.*, 1985) (Fig. 4B), suggesting an effect on internal Cd accumulation in the root tissue.

Strategy II plants suffering from Fe deficiency release the chelating compounds, known as PSs, in the rhizosphere. Given that all PSs are derived from nicotianamine (NA) with methionine and S-adenosylmethionine (SAM) as precursors (Mori and Nishizawa, 1987), it is reasonable that low availability of S could reduce the plant's capability to respond to Fe deficiency (Astolfi *et al.*, 2006a). In the present study, it could be confirmed that the rate of PS release was markedly decreased by S deficiency, reaching

values up to 3-fold lower than control (+S) plants (Fig. 8). In addition, it was found that PS release was further reduced after plant exposure to Cd. The inhibitory effect of Cd followed a similar pattern in all Fe-deficient plants, irrespective of the S supply. Cd exposure has been shown to stimulate PS (DMA) release from maize roots (Hill *et al.*, 2002), although this effect was not associated with a higher Fe uptake (Meda *et al.*, 2007). In the present work, it was found that both S starvation and Cd exposure can qualitatively modify the PS composition. In particular, in Cd-untreated plants, only epiHMA has been detected in root exudates, while the presence of DMA, MA, and epiHMA was revealed in root exudates collected from plants treated with Cd for 24 h. In addition, a complete lack of DMA was found in root exudates collected after 24 h or 72 h of Cd treatment.

There are several reports demonstrating that *HvYSL* and *HvIDS2* genes, encoding a specific Fe(III)–PS transporter (Murata *et al.*, 2006; Harada *et al.*, 2007) and an enzyme responsible for the last step in the production of the MA family of PSs (Nakanishi *et al.*, 2000), respectively, are up-regulated in Fe-deficient plants (Hodoshima *et al.*, 2007). This behaviour was confirmed in the present work (Fig. 9). A progressive increase in transcript abundance of *HvYSL* and *HvIDS2* genes was also found in Fe-sufficient plants exposed to Cd, although no PS release could be detected. An increase in expression of the two genes was also observed when plants were subjected to S deprivation, although, at least for *HvIDS2*, at a lower level. A discrepancy between uptake of the Fe–PS complex and gene expression of the transporter (*ZmYSL*) has been reported in Fe-deficient maize plants treated with Cd (Meda *et al.*, 2007) and tentatively explained on the basis of a direct inhibition of the uptake system by the heavy metal; this, in turn, might have led to a decreased Fe concentration of the tissue and a concomitant stimulation of PS (DMA) release. An effect on the functioning of the Fe–PS transport system might also explain the results of the present study; on the other hand, since PS release was also decreased, some effects on this latter mechanism might be also hypothesized.

In conclusion, the data show that adequate S availability can alleviate Fe deficiency and Cd toxicity in barley plants; on the other hand, it appears that in Fe-deficient plants grown in the presence of Cd with limited S supply, sulphur may be preferentially used in the pathway for biosynthesis of PSs, rather than for production of PCs.

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